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of the Army position, policy or decision unless so designated by other documentation.

Key Accomplishments

1. An abstract was prepared, submitted and accepted for presentation at the 27th Annual International Society for Heart Research American Section Meeting in New Orleans, Louisiana, May 12-15, 2005.
2. Zheng Jingang, Zambraski Edward, Liang Bruce T. Adenosine A_1 receptors mediate potent anti-ischemic skeletal muscle protection in a mouse hindlimb model. J. Mol. Cell. Cardiol. 38(5): 64, 2005.
3. Zheng Jiangang, Zambraski Edward, Liang, Bruce T. Adenosine A_3 Receptors Mediate Potent Anti-ischemic Protection in a Mouse Hindlimb Model of Skeletal Muscle Ischemia and Reperfusion. Manuscript in preparation, 2005-2006

Reportable Outcomes

1. Development of a novel skeletal muscle ischemia/reperfusion injury model, based on mouse hindlimb ischemia/reperfusion, that allows reproducible quantitation of muscle cell death;
2. There has been no patent or intellectual property application at the present time;

Appendices: None

Supporting Data

In the original application, we proposed to use two methods to determine the extent of skeletal muscle injury. The first method uses the NBT dye, nitroblue tetrazolium dye, to distinguish between live and injured muscles. In brief, the muscle flap was sectioned transversely into 1 mm thick segments before staining in 0.033% dye solution in the presence of 1.9 mM NADH. The viable area stained blue/purple as the mitochondrial enzyme activity yields a blue reaction product and the non-viable area will not be stained. The areas can be quantified using an automated image analysis program (Image-Pro Plus, version 5.0, Media Cybernetics, Inc, Silver Spring, MD). Infarct size is calculated as amount of infracted area divided by the total area (blue stained NBT-positive viable area plus the NBT-negative area) in percentage. We were able to show that NBT dye can distinguish between the live vs. injured areas (Fig. 1).

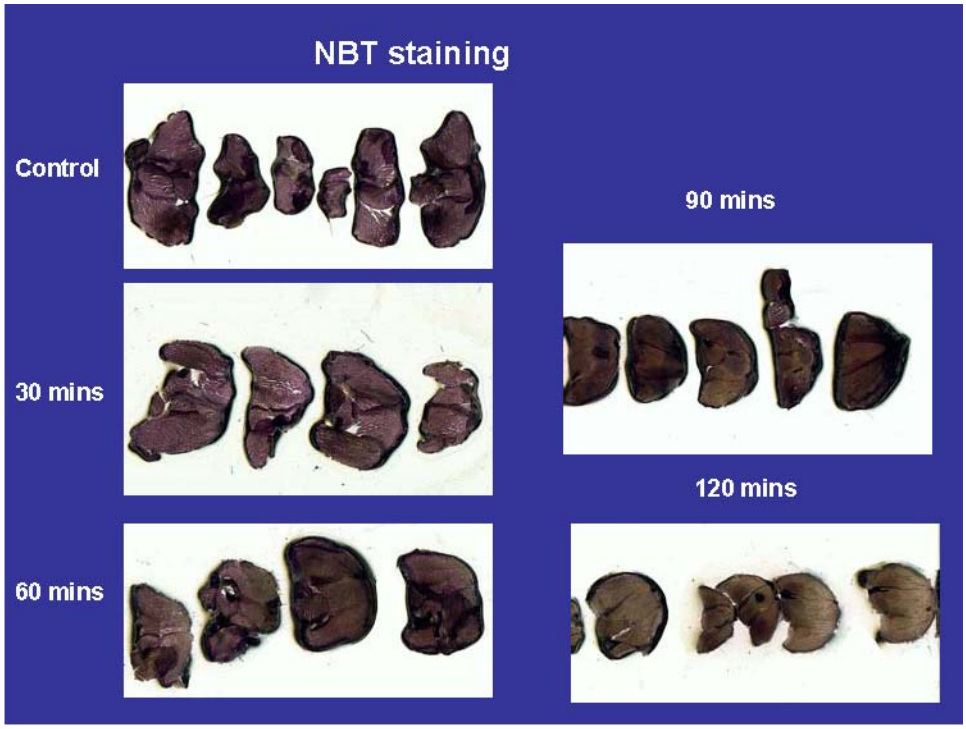
Fig. 1

This section was obtained after 90-min ischemia and 24 hr reperfusion, which was stained with NBT dye. Note the loss of the blue-purple staining in much of the area. The grey area with loss of the muscle appearance is evident.

In contrast, the contralateral muscle without ischemia showed preservation of the muscle appearance with clear blue/purple NBT-stained area indicating viable muscle.

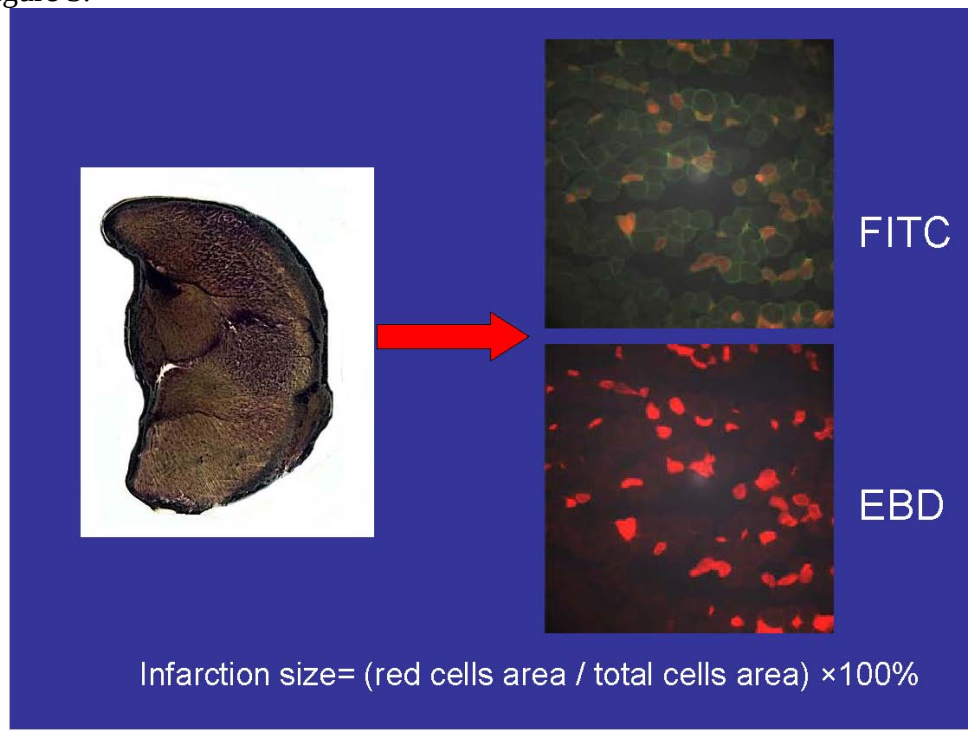
However, this method may not be able to quantify relatively small changes in the extent of skeletal muscle injury that can occur with, for example, different ischemic times. In fact, we had difficulties quantifying the extent of muscle injury during varying ischemic times (see Fig 2 below).

Fig. 2



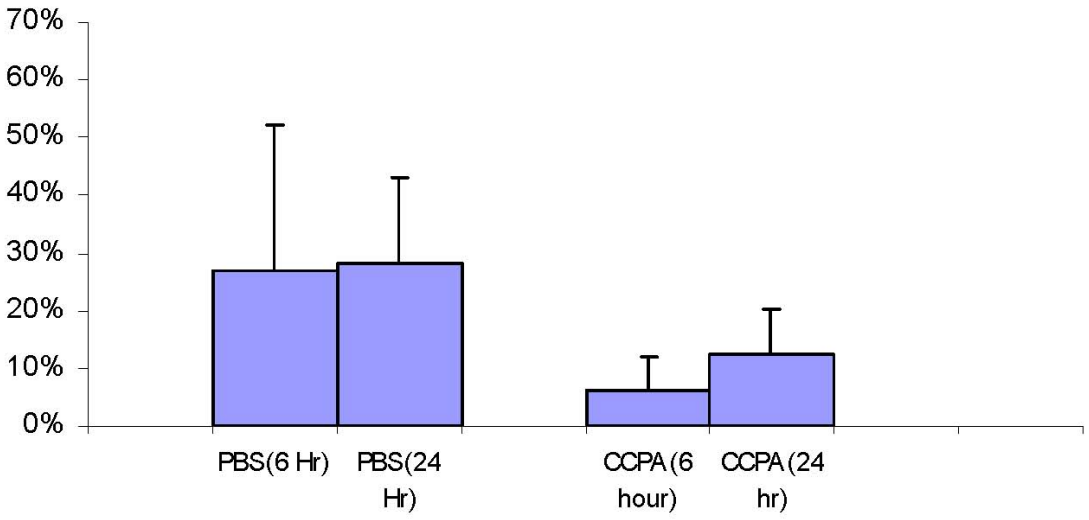
As a result, we developed another method of quantifying skeletal muscle injury, that of using the Evans Blue Dye (EBD). EBD is taken up by injured muscle cells due to permeabilization of the plasma membrane during ischemia/reperfusion. Figure 3 below shows that following ischemia/reperfusion of the gastrocnemius muscle, both NBT and EBD methods were used. The total muscle area of each section was identified by staining with rabbit polyclonal anti-skeletal muscle actin antibodies (ab15265, Abcam Inc, Cambridge, Ma) and goat polyclonal anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC). The injured area was identified by EBD-positive stained area. The percent necrosis or injury was then quantified by obtaining a ratio of two areas.

Figure 3.



Next, we used the EBD method to investigate the protective anti-ischemic effect of adenosine. Figure 4 showed that the adenosine A1 receptor-selective agonist CCPA (2-chloro-N^6-cyclopentyladenosine), when administered 2 hrs before a 90-min ischemia/6 hr or a 90-min/24 hr reperfusion period, was able to decrease the % necrosis.

Figure 4.



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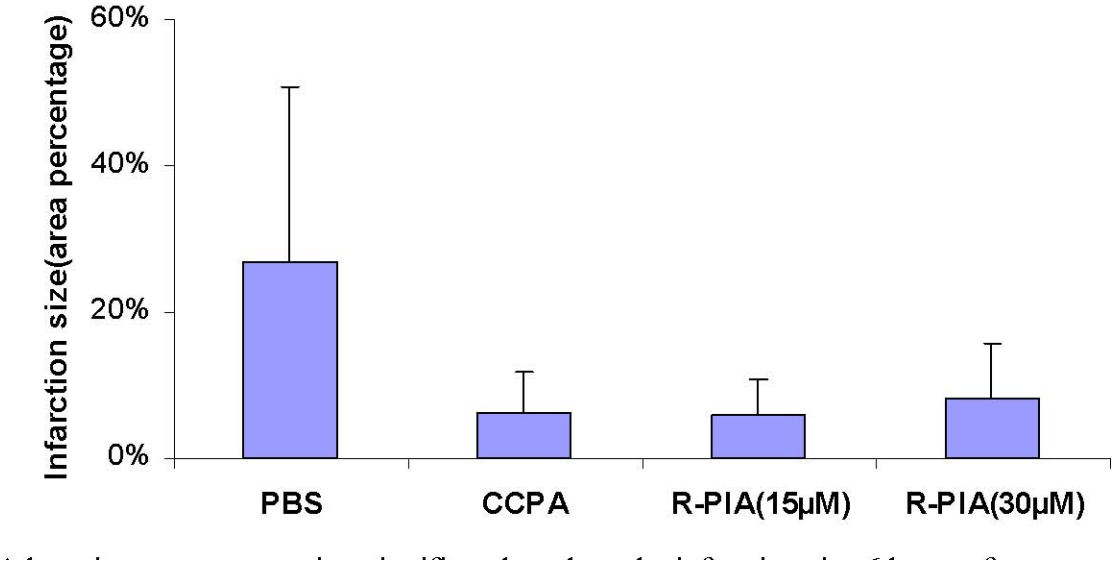
Data were plotted as % EBD-positive cells. The reduced infarction size of the mouse hindlimb skeletal muscle by CCPA remained significant 24 hours after reperfusion in this model. CCPA, 0.1 ml of a 30 μM solution, was given ip per 25 gm of body weight. CCPA vs. PBS, $P<0.05$.

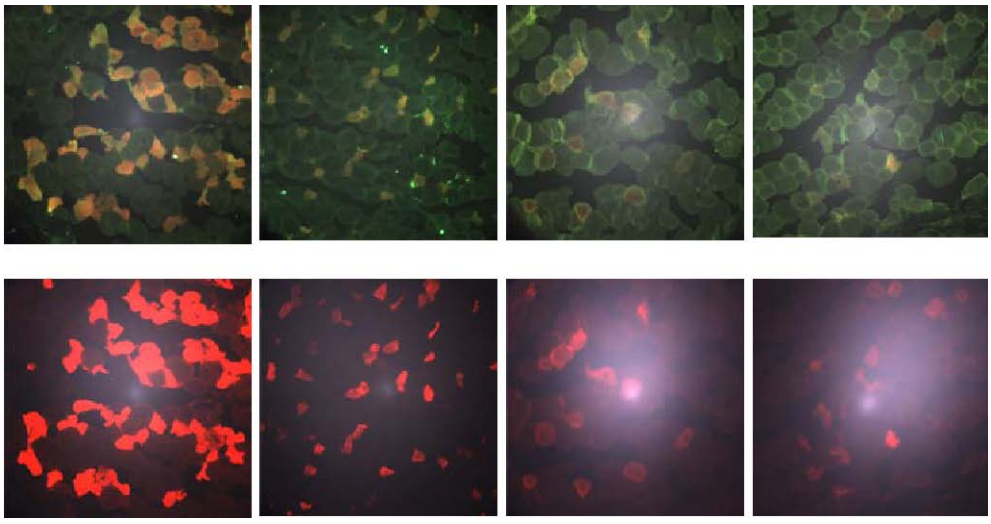
In the next series of experiments (summarized in Figure 5A and B), another adenosine receptor agonist, R-PIA, was tested. R-PIA, N^6-R-phenyl-2-propyladenosine, can activate both adenosine A1 and A3 receptors.

Figure 5A



Adenosine receptor agonists significantly reduce the infarction size 6 hours after reperfusion in mouse hindlimb ischemia reperfusion model. PBS vs any drug: one-way ANOVA, $P<0.05$.

Figure 5B



PBS CCPA (30 μM) R-PIA (30 μM) R-PIA (15 μM)

Top panel: FITC staining; Bottom panel: EBD staining.

Taken together, these data established a novel model of skeletal muscle ischemia/reperfusion injury that allows reproducible quantitation of muscle injury and showed that adenosine receptor activation can exert a potent cytoprotective effect in skeletal muscle.